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RESEARCH PAPER

Metabolic effects of carvedilol through β-arrestin proteins: investigations in a streptozotocin-induced diabetes rat model and in C2C12 myoblasts

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Background and Purpose: Carvedilol is a third-generation β-adrenoceptor antagonist, which also stimulates β-arrestins. β-arrestins initiate intracellular signalling and are involved in insulin release and sensitivity. Carvedilol is superior in effectiveness to other drugs that are used for similar indications and does not cause insulin resistance or diabetes, which can occur with other β-antagonists. We have shown that carvedilol increased glucose usage in C2C12 cells. We investigate the biased agonist efficacy of carvedilol on β-arrestins.

Experimental Approach: Streptozotocin (STZ)-induced diabetes rat model was used to induce metabolic and cardiac disorders. After 8 weeks of diabetes, animals were treated with carvedilol or vehicle for another 4 weeks. In vitro heart function was evaluated at baseline as well as with increasing concentrations of isoprenaline. Effects of diabetes and carvedilol treatment on β-arrestins, ERK, PPARα, CD36 proteins and pyruvate kinase activity were evaluated. β-arrestins were silenced in C2C12 cells by using siRNA. Acute effects of carvedilol on ERK, CD36, mitochondrial transcription factor A, cardiolipin proteins and citrate synthase activity were investigated.

Key Results: Carvedilol reversed the deterioration of cardiac function in diabetes and diabetes-induced decrease in β-arrestins in rats. Carvedilol decreased the expression of CD36 in diabetes and increased mitochondrial transcription factor A and cardiolipin proteins. Silencing of β-arrestins in cells prevented the effects of carvedilol on these proteins.

Conclusion and Implications: The metabolic effects of carvedilol seem to be related to biased activation of β-arrestins. Patients with cardiovascular and metabolic disorders may benefit from new compounds that selectively act on β-arrestins.

KEYWORDS

beta arrestin, biased agonist, carvedilol, substrate metabolism

Abbreviations: CD36, cluster of differentiation 36; ±dP/dt, rate of left ventricular pressure contraction/relaxation; HRP, horseradish peroxidase; IP3, inositol trisphosphate; LVDP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; STZ, streptozotocin; TBS-T, tris-buffered saline containing 0.1% Tween.

1 | INTRODUCTION

Carvedilol is a third-generation β-adrenoceptor antagonist that blocks α_1, β_1 and β_2-adrenoceptors. It is indicated for the treatment of heart failure, hypertension and for the prevention of ventricular dysfunction that can develop after myocardial infarction. It is not only superior in effectiveness to other drugs that are used to prevent myocardial infarction and cardiovascular mortality (Kopecky, 2006; Poole-Wilson et al., 2003) but is also preferable because it does not cause metabolic disorders such as insulin resistance or diabetes, which can occur with other cardiac- or nonselective β-antagonists for chronic use (Lithell, Pollare, & Vessby, 1992; Pollare, Lithell, Selinus, & Berne, 1989; Samuelsson et al., 1994). Moreover, carvedilol was reported to improve insulin sensitivity and serum lipid profile (Jacob et al., 1996) and reduce the risk of diabetes (Torp-Pedersen et al., 2005). Earlier studies reported anti-oxidant, anti-inflammatory, anti-proliferative and anti-arrhythmic properties were involved in its beneficial actions (Calo, Semplicini, & Davis, 2005; Naccarelli & Lukas, 2005; Yue et al., 1992).

Carvedilol is one of the few β-antagonists indicated for heart failure. Currently, the exact mechanism of action for this indication is unknown. However, reduction of heart rate, upregulation of β-adrenoceptors and reduction of unwanted catecholamines effects are amongst the contributing factors (Katzung, 2015). Prevention of increased fatty acid usage due to enhanced sympathetic discharge and increased levels of circulating fatty acids has also been suggested to play a role (Paolisso et al., 1994). To investigate this, we compared direct effects of carvedilol, propranolol, bisoprolol and prazosin on substrate metabolism and showed that both carvedilol and prazosin stimulated glycolysis and decreased fatty acid oxidation in cell culture, whereas the other two drugs had no effect on substrate usage (Onay-Besikci, Suzmecelik, & Ozcelikay, 2012).

On the other hand, Wisler et al. (2007) has shown that carvedilol activates extracellular signal-regulated kinase (ERK) via β-arrestin proteins, while having a negative efficacy for Gs-dependent adenylate cyclase activation. They hypothesized that carvedilol's unique effectiveness in heart failure and other cardiovascular diseases might be related to this so-called biased agonist activity. Luttrell et al. further analysed this biased mechanism of carvedilol and concluded that in addition to recruiting β-arrestins, carvedilol recruits Gi to the β_1 adrenoceptors, but not to the β_2-adrenoceptors (Luttrell et al., 2018). In this respect, β-arrestin proteins, independent of G-protein activation, were shown to protect mice hearts that are challenged with catecholamines (Noma et al., 2007).

Using several models, Luan et al. (2009) reported a strong correlation between β-arrestins and insulin resistance. They showed that β-arrestin2 was downregulated in db/db and dietary-induced insulin-resistant mice as well as from clinical liver samples from type II diabetics. Later, Zhuang, Hu, Zhang, et al. (2011) investigated serum lipids, leptin and glucose tolerance in β-arrestin1 transgenic and β-arrestin1 knockout mice and suggested that knockout mice were more prone to diet-induced obesity and that β-arrestin1 lowered the risk for this disease. The same group also showed that β-arrestin1 modulated transcription through peroxisome proliferator-activated receptor-γ (PPAR-γ; NR1C3) (Zhuang, Hu, Xin, et al., 2011). These studies indicated that β-arrestins may be involved in the insulin effect/sensitivity and suggest that carvedilol's metabolic actions might be related to its bias for this intracellular path.

What is already known

- Carvedilol is a biased agonist through β-arrestins and is beneficial in diabetic cardiomyopathy.

What this study adds

- Carvedilol normalized diabetes-induced alterations in β-arrestin, CD36 and mitochondrial biogenesis.

What is the clinical significance

- Metabolic effects will add to carvedilol's advantages in diabetes and other diseases with cardiovascular/metabolic components.

Beneficial effects of carvedilol has led many researchers to investigate this compound in animal models of diabetes. For example, Grimm et al. (2002) reported that carvedilol treatment for 4 weeks reduced the hyperglycaemia and prevented diabetes-induced increase in extracellular matrix proteins in streptozotocin (STZ)-induced diabetic rats. Carvedilol treatment was later shown to improve higher left ventricular end-diastolic pressure (LVEDP) and lower maximal rate of left ventricle pressure development and decline ($\pm dP/dt_{max}$) in STZ-induced diabetic rats (H. Huang et al., 2007). Treatment of STZ-induced diabetic rats with carvedilol prevented the inhibition of endothelium-dependent relaxation and the decrease of serum nitric oxide (NO) levels caused by diabetes (Fu et al., 2007). However, metabolic effects of carvedilol treatment or a possible link between these effects and β-arrestins were not been investigated in diabetes. We hypothesized that the preferential effects of carvedilol on insulin sensitivity and diabetes, in addition to its superiority amongst other cardiovascular drugs might be attributed to its biased agonist efficacy on β-arrestins. First, a type I diabetes model was utilized to induce metabolic and cardiac disorders and some of these diabetic animals were treated with carvedilol. Next, β-arrestin1 or β-arrestin2 were silenced in C2C12 cells and the metabolic consequences were investigated with or without carvedilol treatment.

2 | METHODS

2.1 | Cell culture and siRNA knockdown of β-arrestin1 and β-arrestin2

C2C12 myoblasts were purchased from T.C. Şap Enstitüsü (Cat#96051501, WDCM number 756, RRID:CVCL_0188) in Ankara,

Turkey. The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) 100 units·ml^{-1} of penicillin and 100 μg·ml^{-1} of streptomycin. Subculturing was performed, using the trypsin method, when the cells reached 80% confluency. Cells were grown at 37°C in a humidified incubator with 5% CO_2.

Silencing of β-arrestin1 or β-arrestin2 was performed using RNA interference (siRNA) technique specific for these β-arrestins (β-arrestin1 [Cat#sc-29742] and β-arrestin2 [Cat#sc-29743]). For transfection, C2C12 cells were plated at 60% confluence in antibiotic-free normal growth medium supplemented with FBS. The siRNA was prepared per manufacturer's specifications and transfected into the C2C12 cells. Concentration of siRNA was determined after confirming the dose dependency of siRNA applications (Figure S1). The final concentration of siRNA transfected into the cells was 70 nM. After 24-h incubation, transfection media were replaced with normal growth medium; 72 h after addition of growth medium, carvedilol (10 μM) or epinephrine (1 μM) was administered for 1 h or 5 min, respectively. The adherent cells were washed with PBS and collected in RIPA lysis buffer and the procedures under the Western blot section were followed, see below.

2.2 | Experimental animals and protocol design

Eight-week-old male Sprague Dawley rats, weighing between 250 and 300 g and obtained from Bilkent University Genetics and Biotechnology Research Center (Ankara, Turkey), were housed in a pathogen-free environment at Ankara University Faculty of Pharmacy Animal Care Unit. This unit operated within a 12-h light/dark cycle at a constant temperature of 22 ± 2°C; and the humidity levels were 30–70%. They were given standard pellet chow (Purina Rat Chow; Optima AS, Turkey) and tap water ad libitum. They were kept in separate cages suitable for rats individually. All animal procedures were approved by Ankara University Animal Care and Use Committee (http://hadyek.ankara.edu.tr/) and performed according to the guidelines from Directive 2010/63/EU of the European Parliament (Approval Number 2016-24-199) as well as the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

Animals were randomly divided into two groups to ensure a similar average body weight. Diabetes was induced by i.p. injection of 40 mg·kg^{-1} of STZ as previously described (Furman, 2015). About 72 h after STZ treatment, blood samples were taken from the caudal vein and random (not fasting) glucose levels were measured with a glucometer (ACCU-CHEK® Performa, Roche Diagnostics, Germany). Rats with glucose levels 3,000 mg·L^{-1} were considered diabetic.

After 8 weeks of diabetes, rats were randomly subdivided into treated and nontreated groups such that diabetic both groups had a similar average blood glucose levels (Figure 1a).

Treatments (with carvedilol or vehicle) started 8 weeks of the induction of diabetes and continued for 4 weeks. Carvedilol was freshly prepared in 1% carboxymethylcellulose, the vehicle. The vehicle was prepared and kept at +4°C for a maximum of 2 days. Carvedilol was freshly prepared by dissolving in the vehicle and given via a feeding needle. Some rats received the vehicle only.

Groups were generated as follows:-

C: control, vehicle-treated, 16 rats

CC: carvedilol (10 mg·kg^{-1}·day^{-1} p.o.) treated control, 16 rats

D: diabetic, vehicle treated, 22 rats

DC: carvedilol (10 mg·kg^{-1}·day^{-1} p.o.) treated diabetic, 22 rats

Treatments were continued for 4 weeks. Blood pressure and heart rate were monitored at third week of the treatment with tail-cuff method (NIBP250, BIOPAC Systems, Inc., Santa Barbara, CA). By the end of treatment, rats were anesthetized with ketamine/xylazine (90/10 mg·kg^{-1}) and sacrificed by exsanguination.

2.3 | Langendorff perfusions

Cardiac function was studied in the isolated heart preparations with the Langendorff technique. Rats were anesthetized by ketamine/xylazine. The hearts were quickly removed and connected to the Langendorff apparatus and perfused at constant pressure with a modified Krebs–Henseleit solution of the following composition (in mmol·L^{-1}): 120 NaCl, 4.8 KCl, 1.25 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$ and 11 glucose (37°C, pH 7.4). The perfusate was equilibrated continuously with a standard 95% O_2/5% CO_2 gas mixture and maintained at 37°C. Heart rate was maintained at 300 beats per minute by right ventricular pacing performed using two electrodes connected to a stimulator (Grass Instrument. Inc., Quincy, MA, USA). A latex balloon connected to a pressure transducer was placed in the left ventricle by polyethylene tubing and the volume in the balloon was adjusted to LVEDP of 10 mmHg. Left ventricular developed pressure (LVDP), rate of left ventricular pressure contraction (+dP/dt) and relaxation (–dP/dt) values were recorded with MP100 (Biopac System, Inc., Santa Barbara, CA).

After 20-min perfusion, baseline values were recorded. Next, cumulative doses of isoprenaline (10^{-11} to 10^{-5} M) were added to perfusion buffer. After being washed, 10^{-5} M of carvedilol was added and the hearts were perfused for another 10 min. Hearts were then removed; left ventricles were excised and snap-frozen in liquid nitrogen.

2.4 | Western blotting

Frozen tissues were pounded in liquid nitrogen and homogenized in ice-cold RIPA buffer that included a protease inhibitor cocktail (100×, Cat#5871, Cell Signaling Technology) and sodium orthovanadate (1 mM, Cat# 450243, Sigma-Aldrich). After sonication, homogenates were centrifuged at 1,300×g for 30 min at 4°C; and the supernatants were used for immunoblotting. The protein concentrations of cell or tissue lysates were measured using the bicinchoninic acid protein assay (Cat#23225, Thermo Fisher Scientific). Equal amounts of protein were separated by SDS-PAGE; 4% acrylamide stacking gel and 8–12% acrylamide separation gels were run on a Mini Protean 2 (BioRad, Hercules, CA) electrophoresis system. The proteins were electrophoretically transferred onto polyvinylidene difluoride or nitrocellulose membrane for 2 h at 100 V. These steps were carried out on ice. To prevent

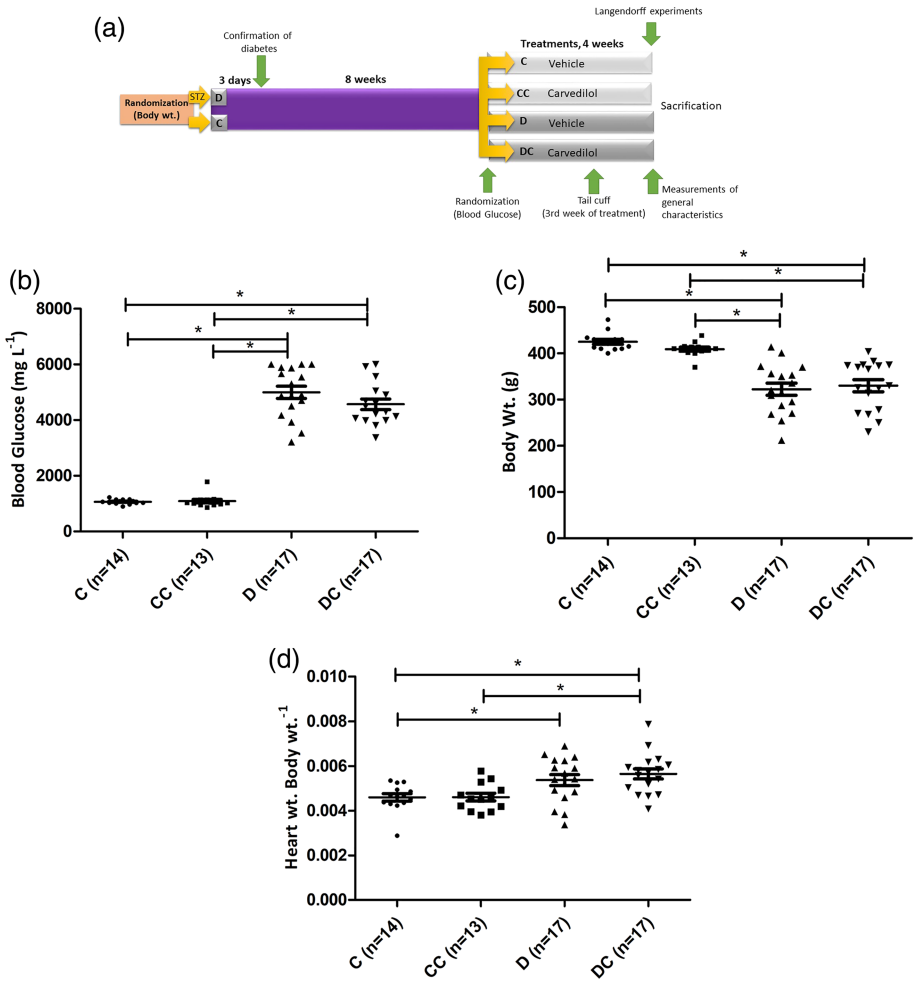


FIGURE 1 Design of animal studies and general characteristics of control and diabetic rats. Experimental timeline (a), blood glucose (b), body weight and (c) the ratio of heart weight to body weight (d) of all groups. C, control; CC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated control; D, diabetic; DC, carvedilol-treated diabetic (*P < 0.05)

nonspecific binding, membranes were incubated with tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 3% BSA for 1 h at room temperature. Membranes were then incubated overnight at + 4°C with primary antibodies. All primary antibodies were of rabbit origin and diluted in TBS-T containing 3% BSA. Membranes were washed for 1 h with TBS and incubated for 1 h at + 4°C with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies. Dilutions of secondary antibody were maintained at 4°C and reused up to six or seven times. After further washing, membranes were incubated with enhanced chemiluminescence (Clarity [Cat#1705061] or Clarity Max [Cat#1705062], BioRad) for 2 min; and chemiluminescent signals were developed on X-ray film (Kodak™). All immuno-related procedures involved comply with the editorial on immunoblotting and immunohistochemistry (Alexander et al., 2018).

After scanning and background subtraction, pictures were analysed with Image J®.

2.5 | Materials

2.5.1 | Antibodies

The antibody of β-arrestin1 (Cat#30036, RRID:AB_2798985, 1:1,000), β-arrestin2 (Cat#3857, RRID:AB_2258681, 1:1,000), pERK1/2-Thr202/Tyr204 (Cat#9101, RRID:AB_331646, 1:2,000), total ERK (tERK)1/2 (Cat#9102, RRID:AB_330744, 1:2,000), GAPDH (Cat#2118, RRID:AB_561053, 1:5,000), β-actin (Cat#4967, RRID:AB_330288, 1:5,000) and rabbit monoclonal antibodies and anti-rabbit IgG secondary antibody (Cat#7074, RRID:AB_2099233, 1:3,000 or 1:5,000) were from Cell Signaling Technology. The polyclonal antibody of α-tubulin (Cat#4074, RRID:AB_2288001, 1:5,000) was from Abcam; and CD36 (Cat#PA1-16813, RRID:AB_568487, 1:1,000), mitochondrial transcription factor A(Cat#PA5-75926, RRID:AB_2719654, 1:1,000) and PPARα (Cat#PA1-32484, RRID:AB_1959487, 1:1,000) polyclonal antibodies were obtained from Thermo Fisher Scientific. Primary antibodies were used at most 10 times, while secondary antibodies were used five times.

A fluorometric assay kit was used to measure cardiolipin content in C2C12 cells mitochondria (BioVision Cat#K944).

2.5.2 | Enzyme activity assays

Pyruvate kinase, hexokinase and phosphofructokinase activities were measured in the left ventricle and skeletal muscle with the **pyruvate kinase**, **hexokinase** and **phosphofructokinase** (PFKFB3) colorimetric assay kits (Sigma-Aldrich, Cat#MAK072, #MAK091 and #MAK092,

respectively). A competitive immunoassay was performed for the quantitative determination of cyclic adenosine monophosphate (cAMP) in the left ventricle (Cat#EMSCAMPL, Thermo Fisher Scientific). Citrate synthase activity was also measured with a colorimetric assay kit (BioVision, Cat#K318) in C2C12 cells. Tissues and cells were homogenized according to the manufacturer's instructions. Reactions were carried out at 37°C.

2.5.3 | Chemicals

Carvedilol was a generous gift from Deva™, Turkey (Product Code 71650, Batch Number 1248). STZ (Cat#S0130) and isoprenaline (Cat#I5627) were from Sigma-Aldrich. Validated siRNA to target β-arrestin1 (Cat#sc-29742), β-arrestin2 (Cat#sc-29743), a scrambled non-silencing siRNA (Cat#sc-37007), siRNA Transfection Reagent (Cat#sc-29528) and siRNA Transfection Medium (Cat#sc-36868) were obtained from Santa Cruz Biotechnology.

2.5.4 | Exclusion criteria

The animals with a blood glucose of less than 3,000 mg·L^{-1} measured 72 h after STZ injection were not included in diabetic groups. To minimize the effects of possible damage during cardiac excision and aortic cannulation, exclusion criteria were applied.

The exact group size for each experimental group is provided in the figure or figure legend and provided in Section 2, which describes the experiment. When used, the term "n" refers to independent values, not replicates. Group sizes at the start were identical, but experimental loss changed the final group size on occasions.

No animal for which the end data were recorded was excluded.

2.5.5 | Statistical analysis

Data are expressed as mean ± SEM. Multiple comparison was conducted if F was significant. The homogeneity of sample variance was confirmed with Bartlett test for all data subjected to ANOVA. Hemodynamic parameters and Western blot studies were analyzed using one-way ANOVA and Bonferroni post hoc tests. Western blots and mitochondrial content assays after β-arrestin silencing were performed using two-way ANOVA for drug effect, followed by Bonferroni post hoc test. A level of probability of $P < 0.05$ was deemed to constitute the threshold for statistical significance. Results with a level of probability of $P < 0.05$ were marked with *. Statistical analysis adheres to the recommendations of the *BJP* on experimental design and analysis in pharmacology (Curtis et al., 2018). The term "control fold mean" indicates that each raw value has been divided by the value of the mean of the controls. Blinding was not feasible owing to the physical characteristics of the animals. Group numbers were determined based on the minimum numbers required for statistical analyses while also considering possible losses during diabetes and treatments. Group size refers to biological samples and not technical replicates. Neither data collection nor data analysis was blinded owing to the rather small study group. Group sizes present the number of independent samples/animals.

2.5.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018) http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2019/20 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019; Alexander, Kelly, et al., 2019).

3 | RESULTS

3.1 | Blood glucose and body weight

Data in Figure 1 were collected at the end of the study after the animals had been killed. Blood glucose levels of untreated and carvedilol-treated (10 mg·kg^{-1}·day^{-1} p.o.) diabetic animals were significantly higher than those of controls (Figure 1b). Diabetic animals lost weight compared to control rats and carvedilol treatment had no effect on body weight (Figure 1c). The ratio of heart weight to body weight is significantly higher in diabetic rats compared with that of control rats (Figure 1d). We have not observed any specific adverse effects related to carvedilol treatment in selected dose.

3.2 | Blood pressure and heart rate

Table 1 shows blood pressure and heart rate of the animals on the third week of treatment. Carvedilol lowered systolic and mean arterial pressures as well as heart rate in non-diabetic rats.

3.3 | Carvedilol improved left ventricular function in diabetic hearts

To evaluate cardiac haemodynamics and myocardial function, baseline parameters (Figure 2) and isoprenaline-induced responses (Figure 3) were assessed using an isolated Langendorff heart preparation. Diabetes deteriorated left ventricular function as indicated by a reduction in baseline LVDP, ±dP/dt and tau values. Carvedilol (10 mg·kg^{-1}·day^{-1} p.o.) treatment significantly improved ±dP/dt values.

In addition, the responsiveness of the hearts to increased demand was assessed by adding increasing concentrations of isoprenaline (10^{-11} to 10^{-5} M) to the perfusate.

At almost every concentration, LVDP and +dP/dt were higher and −dP/dt were lower in the hearts from carvedilol-treated diabetic rats than in untreated diabetic rat hearts (Figure 3). The parameters

TABLE 1 Hemodynamic parameters of control, diabetic and carvedilol (10 mg·kg^{-1}·day^{-1} p.o)-treated rats at the third week of treatment

Parameter	Group			
	Control (C)	Carvedilol-treated control (CC)	Diabetic (D)	Carvedilol-treated diabetic (DC)
Systolic BP (mmHg)	161.9 ± 4.9 *n* = 12	145 ± 5.6 *n* = 11	155.2 ± 5.1 *n* = 16	146.6 ± 4.3 *n* = 15
Diastolic BP (mmHg)	143 ± 4.3 *n* = 12	145.1 ± 4.1 *n* = 11	119.3 ± 2.9[a] *n* = 16	124.7 ± 4.8[a] *n* = 15
Mean arterial pressure (mmHg)	160.5 ± 4.4 *n* = 12	135.9 ± 5.2[a] *n* = 11	142 ± 4.8 *n* = 16	138.4 ± 4.8[a] *n* = 15
Heart rate (beats per min)	375.3 ± 7.1 *n* = 12	339.4 ± 7.6[a] *n* = 11	305.5 ± 8.5[a] *n* = 16	287.8 ± 8.3[a] *n* = 15

Data are presented as mean ± SEM. $P < 0.05$.
[a]vs. C.

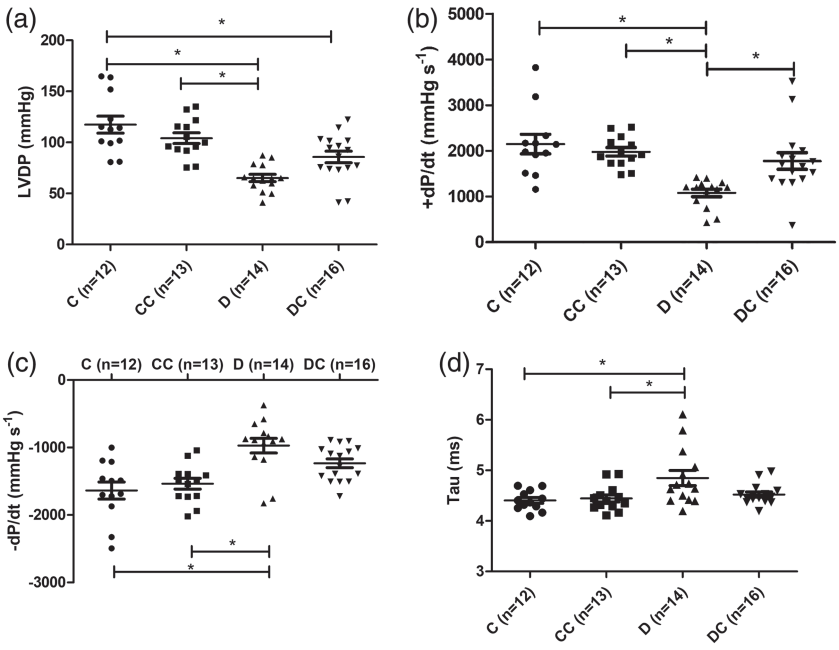


FIGURE 2 Baseline parameters. LVDP (a), +d*P*/d*t* (b), −d*P*/d*t* (c) and tau (d) of the left ventricle. Heart rate was maintained at 300 beats per minute. The latex balloon was adjusted to LVEDP of 10 mmHg. Values were recorded after 20-min perfusion at constant pressure in Langendorff. C, control; CC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated control; D, diabetic; DC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated diabetic; LVDP, left ventricular developed pressure (*$P < 0.05$)

obtained with the addition of 10^{-5} M were not significantly different between groups and therefore not included in Figure 3. Similarly, at nearly all concentrations of isoprenaline, the difference in LVDP and ±d*P*/d*t* between carvedilol-treated diabetic hearts and control hearts was also not significant.

3.4 | Effects of diabetes and carvedilol treatment on β-arrestins

To explore STZ diabetes and carvedilol-mediated effects, the expressions of β-arrestins were determined by Western blot in both the ventricles and skeletal muscles. β-arrestin1 was not affected by diabetes or carvedilol treatment in the left ventricle (Figure 4a). However, diabetes diminished β-arrestin1 levels in the skeletal muscle, which was normalized by carvedilol treatment (Figure 4c). β-arrestin2 protein was also reduced by diabetes in the left ventricle and skeletal muscle and this decrease was reversed by carvedilol treatment in both tissues (Figure 4b,d).

3.5 | Effects of diabetes and carvedilol treatment on ERK and cAMP levels

Carvedilol triggers ERK phosphorylation through β-arrestins (Wisler et al., 2007). To further establish the interaction between carvedilol and β-arrestin-mediated events, carvedilol's effect on ERK phosphorylation was examined in ventricles (Figure 5a). In addition, carvedilol (10^{-5}M) was administered acutely to untreated control and diabetic rat hearts at Langendorff setting (Figure 5b).

Diabetes significantly increased ERK phosphorylation in the left ventricle and carvedilol treatment abolished this effect (Figure 5a).

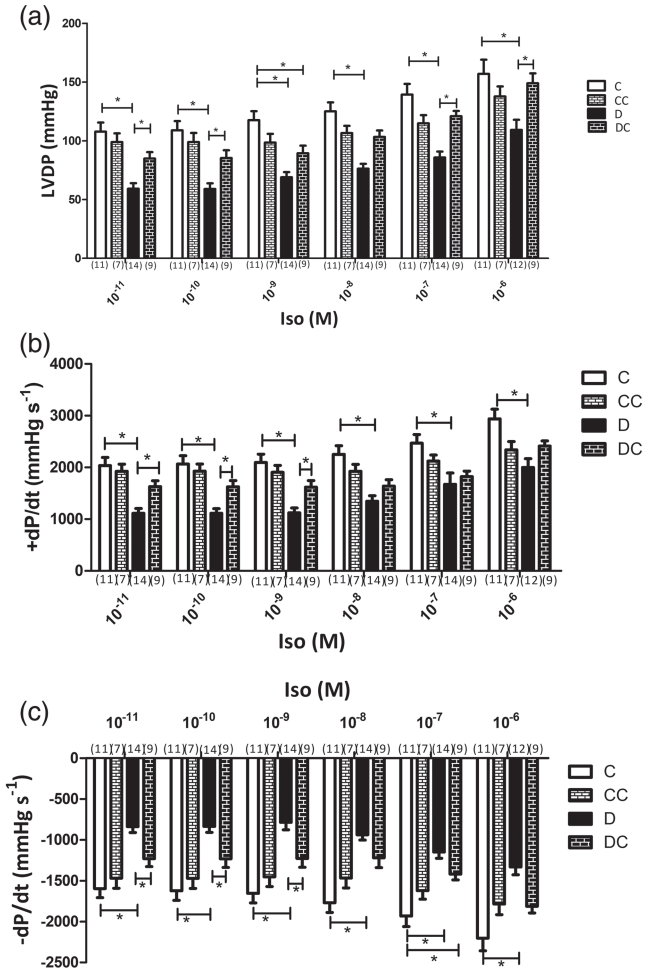


FIGURE 3 Comparison of inotropic responses to isoprenaline challenge. LVDP (a), +d*P*/d*t* (b), and −d*P*/d*t* (c) of the left ventricle. After 20-min perfusion at constant pressure in Langendorff, cumulative doses of isoprenaline (10^{-11} to 10^{-5} M) were added to perfusion buffer. The size of each group is depicted in parentheses. C, control; CC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated control; D, diabetic; DC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated diabetic; LVDP, left ventricular developed pressure (*P < 0.05)

Interestingly, acute administration of carvedilol caused a slight increase of ERK phosphorylation in control rat hearts (Figure 5b). tERK normalized to β-actin (Figure 5c) and cAMP levels (Figure 5d) were similar in all groups.

3.6 | Carvedilol effect on metabolic pathways

As an indicator of fatty acid metabolism, the protein expression levels of CD36, also known as the fatty acid translocase, were evaluated in the left ventricle (Figure 6a), skeletal muscle (Figure 6b) and liver (Figure 6c) of the animals. Diabetes increased CD36 protein expression in these tissues. Carvedilol treatment significantly decreased CD36 to control levels.

Peroxisome proliferator-activated receptor-α (PPARα; NR1C1) expression was also assessed as a marker of fatty acid metabolism in the skeletal muscle and left ventricle. Like CD36, diabetes increased PPARα expression in the skeletal muscle and this was prevented by carvedilol treatment (Figure 6c). A similar pattern was observed in the left ventricle; however the alterations were not statistically significant (data not shown).

Glucose usage was evaluated by investigating the activities of the key enzymes involved in carbohydrate metabolism in both the left ventricle and skeletal muscle of all groups. As expected, diabetes reduced pyruvate kinase activity in the left ventricles (Figure 7a). As an indicator of improved glucose usage, carvedilol treatment improved pyruvate kinase activity in diabetic animals. A similar trend was observed in the skeletal muscles of the animals (Figure 7b). None of these alterations were evaluated statistically owing to limited n number. Therefore, this data is deemed preliminary by the *BJP* and will be reconsidered for further analysis.

3.7 | Involvement of β-arrestins on substrate metabolism and mitochondrial biogenesis: Studies on cells

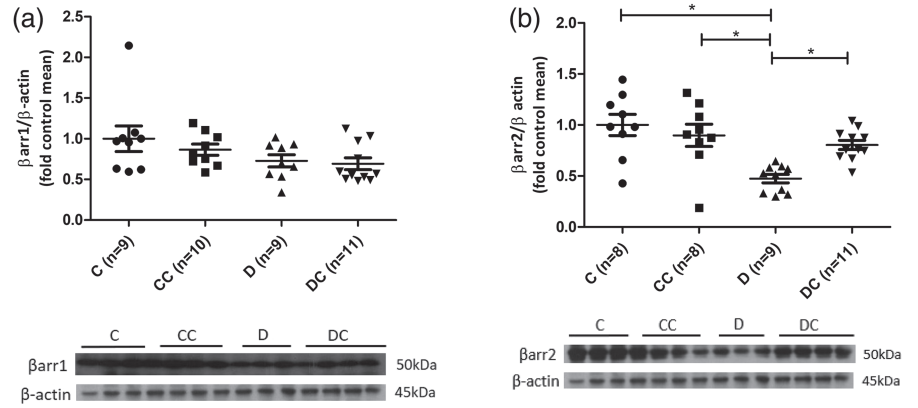
Silencing of β-arrestins was confirmed by Western blot analysis (Figure 8). Both β-arrestin1 and β-arrestin2 were successfully silenced as shown by a nearly 50% reduction of protein expressions. Next, silencing was confirmed by the reduction of ERK phosphorylation in β-arrestin1-silenced (Figure 8c) and β-arrestin2-silenced (Figure 8d) cells. As expected, carvedilol (10μM) administration did not trigger ERK phosphorylation in silenced cells to the same level as non-silenced control cells. The effect through G-proteins was ruled out by the lack of effect of carvedilol on second messengers of Gs and Gq (Figure S2).

C2C12 cells were incubated with carvedilol (10μM) for increasing lengths of time (5–120 min) in order to determine carvedilol-triggered ERK phosphorylation as a downstream effector of β-arrestins. ERK phosphorylation was significantly higher in cells that were incubated with carvedilol for 60 min (Figure S3A).

3.8 | Carvedilol's effect on CD36 and mitochondrial transcription factor A expressions and the involvement of β-arrestins

To investigate the involvement of β-arrestins on substrate metabolism and mitochondrial biogenesis, carvedilol (10μM) was administered on β-arrestin1- or β-arrestin2-silenced cells and their scrambled controls. Carvedilol significantly decreased the expression of CD36,and this was not observed in β-arrestin1-silenced cells (Figure 9a). A similar decrease was seen in β-arrestin2-silenced cells, however this was not statistically significant (Figure 9b).

Moreover, carvedilol increased the expression of mitochondrial transcription factor A, a transcription factor involved in mitochondrial



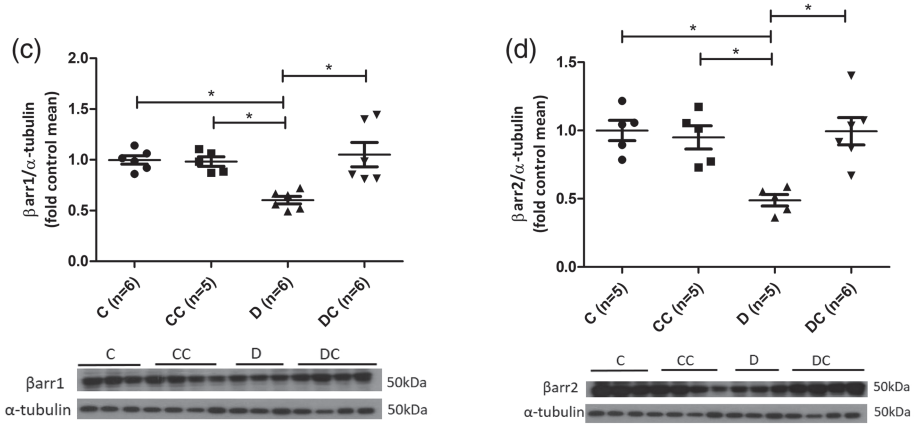
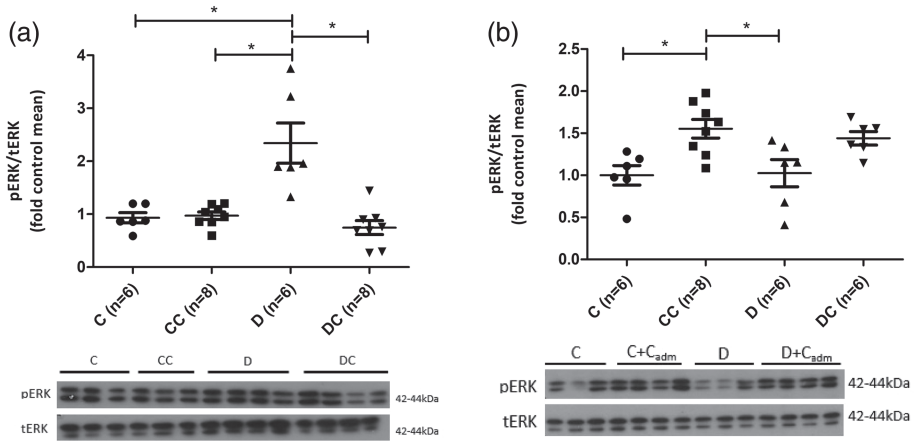


FIGURE 4 β-arrestin (βarr)1 and βarr 2 expression. βarr1 (a) and βarr2 (b) in the left ventricles and βarr1 (c) and βarr2 (d) in the skeletal muscles. Values were normalized to β-actin or α-tubulin and representative Western blot images are shown. C, control; CC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.)-treated control; D, diabetic; DC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.) -treated diabetic (*P < 0.05)



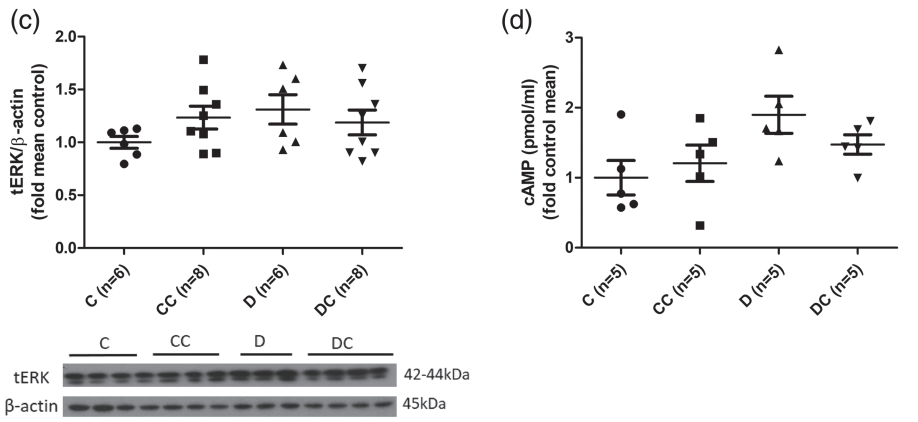


FIGURE 5 ERK phosphorylation and cAMP levels. The effect of carvedilol-treatment (a) and acute carvedilol administration in Langendorff (10^{-5} M, 10 min) (b) on ERK phosphorylation. Phosphorylated ERK values were normalized to total ERK protein. The effect of diabetes and carvedilol-treatment on tERK (c) and cAMP levels (d) in the left ventricle. Representative Western blot images are shown. C, control; CC, carvedilol(10 mg·kg^{-1}·day^{-1} p.o.) -treated control; cAMP, cyclic adenosine monophosphate; D, diabetic; DC, carvedilol (10 mg·kg^{-1}·day^{-1} p.o.) -treated diabetic (*P < 0.05)

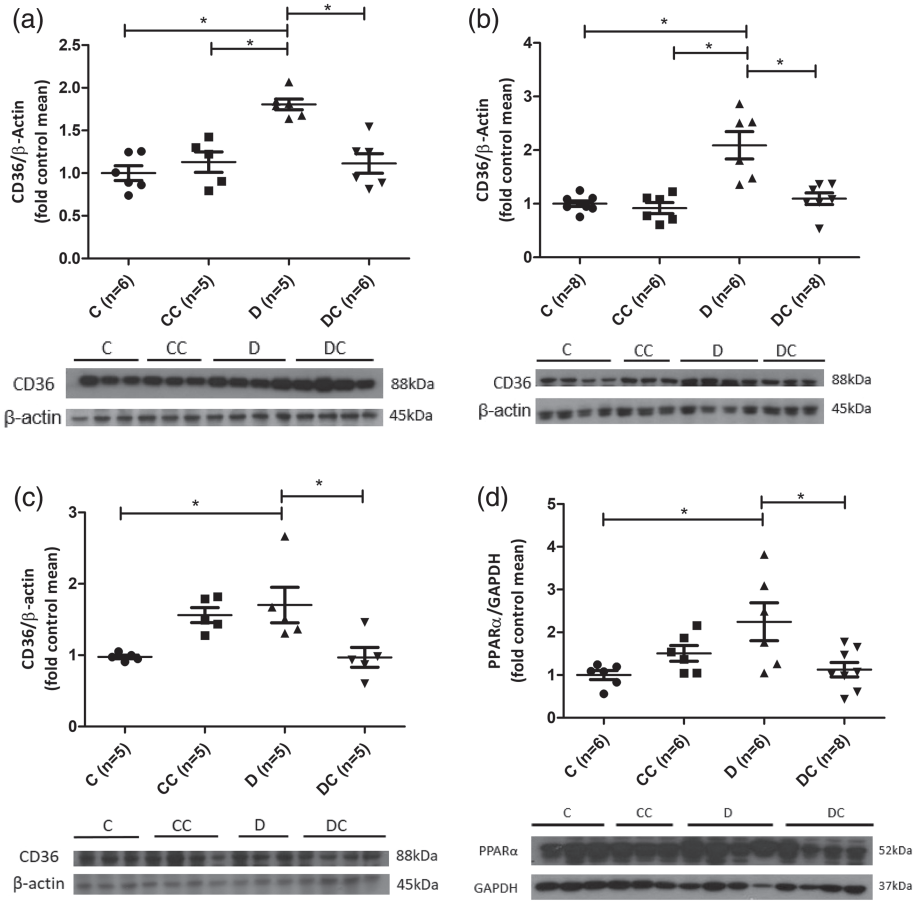


FIGURE 6 Proteins involved in fatty acid metabolism. CD36 levels in the left ventricle (a), skeletal muscle (b), and liver (c) and PPARα levels in the skeletal muscle (d). Values normalized to β-actin or GAPDH and representative Western blot images are shown. C, control; CC, carvedilol (10 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o.)-treated control; D, diabetic; DC, carvedilol (10 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o.) -treated diabetic (*$P < 0.05$)

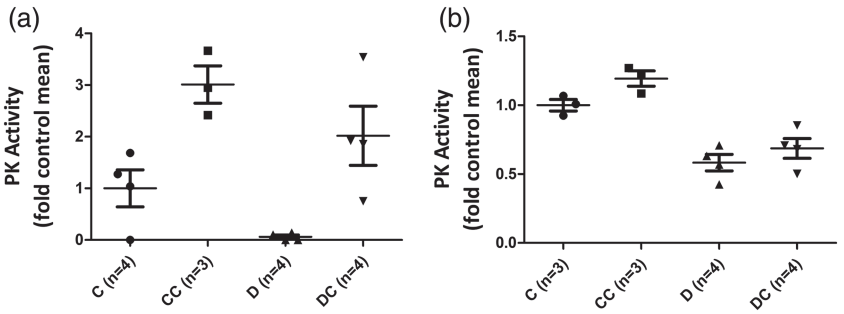


FIGURE 7 Evaluation of pyruvate kinase (PK) activity. Assessment in the left ventricle (a) and skeletal muscle (b) in all groups. C, control; CC, carvedilol (10 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o.)-treated control; D, diabetic; DC, carvedilol (10 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o.) -treated diabetic

biogenesis, in the presence of β-arrestin proteins (Figure 9c,d). This effect was not observed when either β-arrestin1 or β-arrestin2 was silenced.

3.9 | Carvedilol's effect on mitochondrial content and the involvement of β-arrestins

In order to evaluate the effects of carvedilol and β-arrestins on mitochondrial content, certain biomarkers were examined. Cardiolipin content and citrate synthase activity are frequently used as biomarkers of mitochondrial content (Larsen et al., 2012).

Carvedilol significantly increased cardiolipin content, which was not observed in β-arrestin1-silenced (Figure 10a) or β-arrestin2-silenced (Figure 10b) cells. Citrate synthase activity was not affected either by carvedilol nor β-arrestin1 (Figure 10c) and β-arrestin2 (Figure 10d) silencing.

4 | DISCUSSION

This study confirms the beneficial effects of carvedilol in diabetic rats by improving baseline hemodynamic parameters. This has been reported previously, where carvedilol treatment prevented early

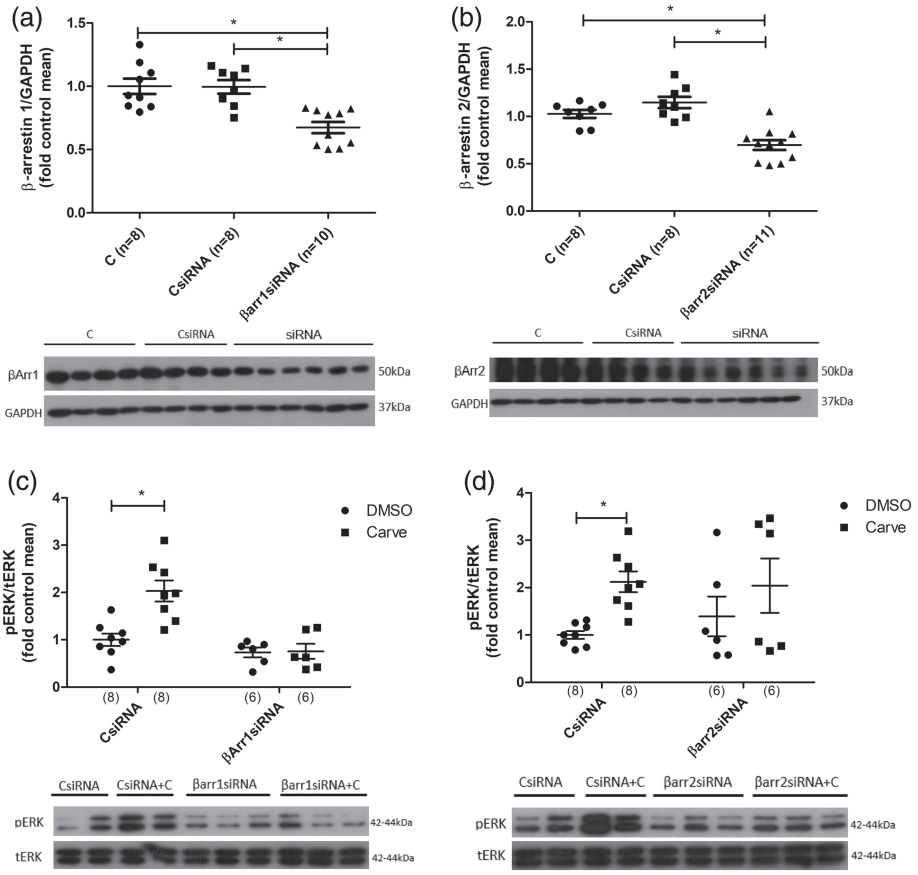


FIGURE 8 Verification of β-arrestin (βarr) silencing. βarr1 (a) and βarr2 (b) protein expression after siRNA transfection; phosphorylated ERK protein levels in βarr1 (c) and βarr2 (d) silenced cells after carvedilolincubation (10μM, 60 min, as determined by supporting experiments and shown in Figure S3A). Values normalized to tERK or GAPDH and representative Western blot images are shown. The size of each group is depicted in parentheses. C, control, no transfection; CsiRNA, scrambled control siRNA; CsiRNA+C, carvedilol-treated scrambled control siRNA; βarr1siRNA+C, carvedilol-treated βarr1siRNA; βarr2siRNA+C, carvedilol-treated βarr2siRNA (*$P < 0.05$)

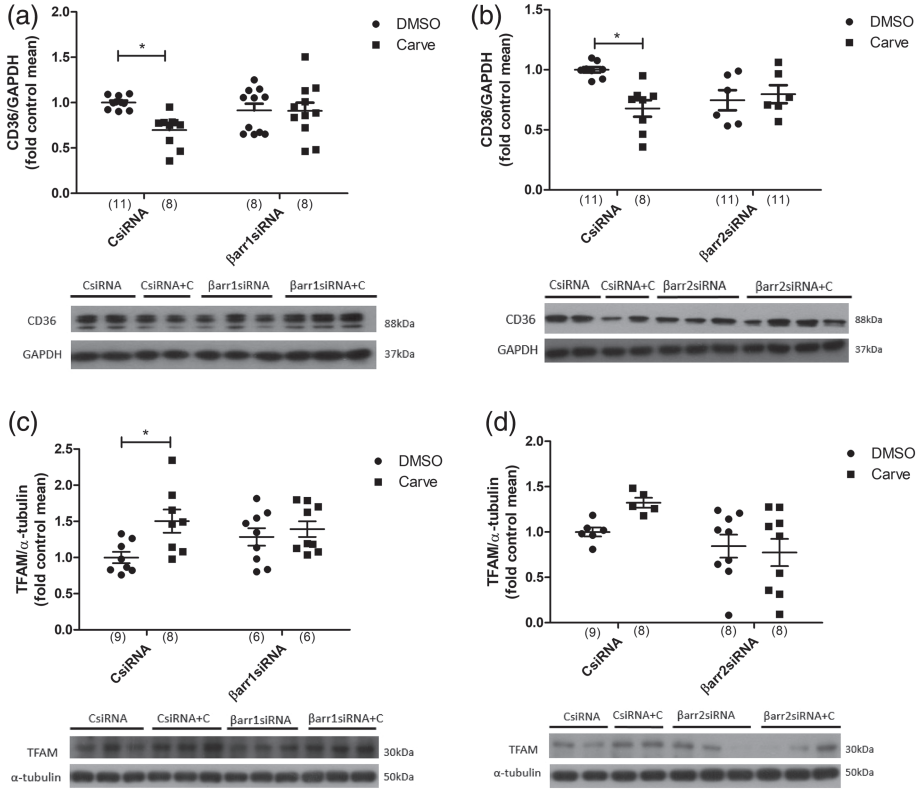


FIGURE 9 Evaluation of the effects of carvedilol (10μM) administration on CD36 and mitochondrial transcription factor A (TFAM) expressions in C2C12 cells. CD36 protein expression in β-arrestin (βarr)1-silenced (a) and βarr2-silenced (b) or scrambled controls; TFAM protein expression in βarr1-silenced (c) and βarr2-silenced (d) or scrambled controls. Values normalized to α-tubulin or GAPDH and representative Western blot images are shown. The size of each group is depicted in parentheses. CsiRNA, scrambled control siRNA; CsiRNA+C, carvedilol-treated scrambled control siRNA; βarr1siRNA+C, carvedilol-treated βarr1siRNA; βarr2siRNA+C, carvedilol-treated βarr2siRNA (*$P < 0.05$)

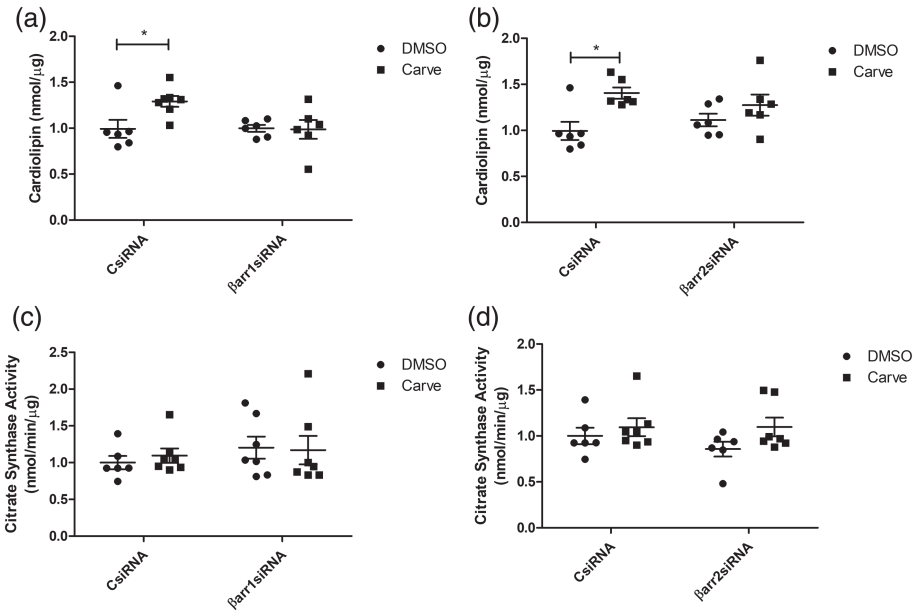


FIGURE 10 Evaluation of the effects of carvedilol (10μM) administration on mitochondrial content parameters in C2C12 cells. Cardiolipin content in β-arrestin (βarr)1-silenced (a) and βarr2-silenced (b) or scrambled controls and citrate synthase activity in βarr1-silenced (c) and βarr2-silenced (d) or scrambled controls (n = 6). CsiRNA, scrambled control siRNA; CsiRNA+C, carvedilol-treated scrambled control siRNA; βarr1siRNA+C, carvedilol-treated βarr1siRNA; βarr2siRNA+C, carvedilol-treated βarr2siRNA (*P < 0.05)

diabetic impairment of basal cardiac functions (H. Huang et al., 2007). In our study, we started with carvedilol treatment 8 weeks after the induction of diabetes simply to get a better understanding of the reversal effect of carvedilol. When compared with controls, STZ-induced model of type I diabetes induced cardiac dysfunction as shown by lower LVDP and ±dP/dt in diabetic hearts. Carvedilol treatment improved basal levels of these parameters. In addition, increasing concentrations of isoprenaline were added during Langendorff perfusion in order to assess the capacity of the hearts to increase metabolic demand (Bombicino et al., 2016; Jain et al., 2001). ±dP/dt were significantly different at every concentration of isoprenaline in diabetic hearts compared with control hearts. Similarly, LVDP values of diabetic hearts were lower at nearly all concentrations of isoprenaline except for 10^{-6} M of the drug. Carvedilol treatment reversed the deterioration of function as shown by similar values of LVDP and ±dP/dt between control and carvedilol-treated diabetic hearts. Nevertheless, LVDP was still significantly lower in diabetic hearts at the isoprenaline concentration of 10^{-6} M and −dP/dt was lower at 10^{-7} M of the drug. Despite these differences at only a few concentrations of isoprenaline, carvedilol seemed to improve cardiac function.

Carvedilol reduced blood pressure in control animals. Even within therapeutic dose range, carvedilol was reported to lower blood pressure in nearly 10% of mild-to-moderate heart failure patients (Packer et al., 1996). This effect was reported to be highest during the first 30 days of dosing in humans. We believe that the reduction of blood pressure with carvedilol in control animals may be related to the dose that we used based on previous reports (H. Huang et al., 2007). Albeit vaguely, a small decrease in diabetic animals treated with carvedilol supports this interpretation.

It is now widely accepted that GPCRs couple not only to G-proteins but also others and, depending on the receptor or the ligand, initiate other signalization pathways. Similarly, β-arrestins, once thought to be responsible for the desensitization and thereby ending GPCR activity, mediate intracellular signalling (Andresen, 2011; DeWire, Ahn, Lefkowitz, & Shenoy, 2007; DeWire & Violin, 2011). In other words, binding of a ligand to GPCR may initiate intracellular signalling via β-arrestins, independent of the G-proteins. This concept of agonist–receptor interaction is known as biased agonism (Andresen, 2011). Accordingly, a ligand may be classified as antagonist if only Gα-Gβγ-mediated intracellular events (and not β-arrestin-mediated signalling) are assessed. Unexpected effects of ligands that were once known as antagonists or different clinical effects of the antagonists, although blocking the same receptor, may be explained by this biased interaction. Until it was shown to initiate β-arrestin-mediated signalling (Wisler et al., 2007), carvedilol was classified as an antagonist of β-adrenoceptors and it is perhaps one of the best examples of this phenomenon. Similarly, another third-generation β-antagonist, nebivolol, was reported to be a β-arrestin-biased agonist (Erickson et al., 2013).

Carvedilol is unique amongst other compounds within the same drug family. It does not impair insulin sensitivity or lead to diabetes like other β-antagonists but seem to improve the metabolic profile as reported by many studies (Jacob et al., 1996; Lithell, Pollare, & Vessby, 1992; Pollare, Lithell, Selinus, & Berne, 1989; Samuelsson et al., 1994; Torp-Pedersen et al., 2005). We thought that the preferential effects of carvedilol might be related to its possible effect on substrate metabolism. To investigate this, we compared direct effects of carvedilol with propranolol, bisoprolol and an α adrenoceptor antagonist, prazosin on substrate metabolism. We showed that both carvedilol and prazosin stimulated glycolysis and decreased fatty acid oxidation (Onay-Besikci, Suzmecelik, & Ozcelikay, 2012). We had not investigated the mechanism of this effect. Today, the exact mechanism of beneficial properties of carvedilol is still not entirely known. However, an intracellular signalling pathway that is preferentially activated by carvedilol may specifically modulate substrate metabolism towards more glucose usage. We believe that carvedilol, via activation

of β-arrestins, lowers fatty acid usage. Interestingly, nebivolol was shown to stimulate mitochondrial biogenesis, which may suggest that biased agonists of β-arrestins have a regulatory role on substrate metabolism (C. Huang, Chen, Xie, Yang, & Shen, 2013; Yao et al., 2016). In this study, we showed that β-arrestin2 protein levels decreased in the skeletal muscle and left ventricles in diabetic rats while β-arrestin1 decreased in the skeletal muscle only. The decrease in β-arrestin2 was accompanied by an increase in CD36 in both skeletal muscle and left ventricles of diabetic rats. Moreover, PPARα was higher in the skeletal muscle of the diabetic rats. Carvedilol treatment of diabetic animals prevented the decrease in β-arrestin2 and increase in CD36 as well as PPARα. Moreover, incubation of scrambled control C2C12 cells with carvedilol lowered CD36 levels and this decrease was prevented when β-arrestin1 was silenced (Figure 9a). This seems to indicate that carvedilol's effect on CD36 is primarily regulated through by β-arrestin1 in the skeletal muscle.

Glucose usage was evaluated by investigating the activities of the key enzymes involved in carbohydrate metabolism in both left ventricle and skeletal muscle of all groups. Although still preliminary, diabetes reduced pyruvate kinase activity in the left ventricles. As an indicator of improved glucose usage, carvedilol treatment improved pyruvate kinase activity in diabetic animals. A similar trend was observed the skeletal muscles of the animals. In addition, the activities of hexokinase and phosphofructokinase were determined in both skeletal muscles and left ventricles of the animals. Diabetes or carvedilol treatment had no significant effect on these enzymes (data not shown). These results indicate that fatty acid metabolism and metabolic modulation of carvedilol are mediated by β-arrestins in this model of diabetes.

Many studies suggested a correlation between diabetes/insulin resistance and β-arrestins. A differential regulation of β-arrestins was reported by other studies. For example, β-arrestin2 protein and mRNA levels were lower in both db/db model of type II diabetes and high fat diet obesity (Luan et al., 2009). β-arrestin1 was less affected in these models. In the same study, glucose utilization was lower and insulin tolerance was impaired in β-arrestin2 knockdown mice. Moreover, administration of β-arrestin2 restored insulin sensitivity. These results indicated that β-arrestin2 was anti-diabetic (Luan et al., 2009). In two studies, Zuang et al. reported that the expression of β-arrestin1 repressed adipogenesis and diet-induced obesity and improved glucose tolerance and insulin sensitivity though interaction with PPARγ. It was reported that PPARγ-mediated expression of metabolic genes required β-arrestin1 binding. The authors concluded that β-arrestin1 played an important role in the prevention of obesity and perhaps other metabolic disorders (Zhuang, Hu, Xin, et al., 2011; Zhuang, Hu, Zhang, et al., 2011). **Glucagon-like peptide-1** (GLP-1) receptors are also a member of the GPCR family and regulate pancreatic insulin release in response to food intake. Using immunoprecipitation in INS-1 cells, Sonoda et al. (2008) showed that β-arrestin1 interacts with **GLP-1 receptor**. Taken together, these studies indicate that β-arrestins serve many functions such as insulin release and sensitivity. Carvedilol's metabolic advantages may be related to its biased agonist activity on these proteins in different tissues.

One of the downstream effects of β-arrestin-activated intracellular process is phosphorylation of ERK and the phosphorylation status of this protein is often used as a marker of biased agonists (Erickson et al., 2013; Wisler et al., 2007). Carvedilol was shown to induce ERK phosphorylation through β-arrestins (Wisler et al., 2007). Both total and phosphorylated levels of ERK were increased in diabetic hearts and returned to control levels with carvedilol treatment (Figure 5a). These findings are somewhat similar to the results by Strniskova et al. (2003), where they showed an increase in ERK phosphorylation after ischemia, but not at basal levels. To analyse this further, carvedilol was added acutely during perfusion of some untreated control and diabetic hearts. Acute administration resulted in an increase in ERK phosphorylation (Figure 5b), which was more pronounced and significant in untreated control hearts. This indicated that the acute effect of carvedilol is stimulation of ERK phosphorylation that also suggests the activation of β-arrestin pathway. Sundgren et al. (2003) reported that the cardiac dysfunction in obesity and insulin resistance is related to an enhanced ERK signalling. In addition, carvedilol was shown to reduce ERK activation in hyperinsulinemia (Wang et al., 2017). When combined with improved function and metabolic alterations, normalizing effect of carvedilol on ERK seems to indicate that the stimulation of ERK phosphorylation may be a compensatory response to a metabolic challenge in our model.

We believe that our results provide the link between carvedilol-induced metabolic effects and β-arrestins. Impairment of mitochondrial biogenesis is accompanied by and even suggested to cause problems related to substrate metabolism in the skeletal muscle and adipose tissue (Choo et al., 2006; Lowell & Shulmanz, 2005; Sivitz & Yorek, 2010). Most metabolic reactions occur in the mitochondria and carvedilol was shown to stimulate mitochondrial biogenesis in endothelial cells (Yao et al., 2016). Mitochondrial biogenesis is primarily regulated by PPARγ coactivator PGC-1α. PGC-1α is an activator for nuclear transcription factors that regulate mitochondrial biogenesis, nuclear respiratory factor (NFR) 1 and 2 (Duncan, Fong, Medeiros, Finck, & Kelly, 2007; Wu et al., 1999). NRF1 activates mitochondrial transcription factor A (Scarpulla, 2002). In the study by Yao et al. (2016), stimulation of mitochondrial biogenesis by carvedilol was accompanied by an increase in PGC-1α, NRF1 and mitochondrial transcription factor A. However, the authors have not investigated the link between β-arrestins and this carvedilol effect. In our study, we showed that mitochondrial transcription factor A was stimulated by incubating the cells with carvedilol and this effect was not observed in β-arrestin1 and β-arrestin2 knocked down cells. Similarly, carvedilol significantly increased cardiolipin content, which was not observed in β-arrestin1 and β-arrestin2-silenced cells, indicating that mitochondrial biogenesis is controlled by both isoforms of β-arrestins.

In two similar studies, Zheng et al. reported that carvedilol reduced diabetic cardiomyopathy in rats by inhibiting diabetes-induced increase in pro-inflammatory cytokines and cardiomyocyte apoptosis and activation of mitochondrial oxidative stress signalling pathway. The authors concluded that they were associated and involved in the pathogenesis of diabetic cardiomyopathy. The effect of carvedilol in control animals was not investigated in this study and

the authors have not linked their findings with GPCR coupling or biased agonist activity of carvedilol (Zheng et al., 2017, 2019).

Carvedilol was recently shown to restore lactate levels in ventromedial hypothalamus induced by recurrent hypoglycaemia in rats and thereby prevented the impairment of hypoglycaemia awareness. In addition, elevated lactate levels in the brain in recurrently hypoglycaemic animals were accompanied by increased mRNA expression of **monocarboxylate transporter**, *Mct2*. Interestingly, *Mct2* expression was reduced with carvedilol treatment along with the reduction in extracellular lactate levels, further suggesting an improved utilization of glucose with the drug (Farhat et al., 2019).

Recently, carvedilol and bisoprolol were compared in heart failure patients for their effects on inflammatory and oxidative stress markers. The authors concluded that both compounds have beneficial effects. They concluded that carvedilol was more effective in reducing oxidative stress, an effect repeatedly shown for carvedilol treatment (Toyoda et al., 2020).

To summarize, the decrease in fatty acid metabolism with carvedilol was shown in our previous work (Onay-Besikci, Suzmecelik, & Ozcelikay, 2012). Moreover, it is well documented that the contractile performance of the heart is greater when the heart is oxidizing more glucose and less fatty acids to produce energy as reviewed by Stanley et al. (2005). Our study showed that even in diabetes, a situation where glucose usage is limited, carvedilol was able to prevent functional deterioration of the hearts. Although limited by the lack of blood/tissue levels of fatty acids, a decrease in CD36 with carvedilol treatment in diabetic hearts indicates that carvedilol decreases fatty acid utilization and stimulates glucose usage and this seem to be mediated by β-arrestin2 in the heart. Moreover, modulatory effect of carvedilol on substrate metabolism is confirmed by a decrease in both β-arrestins that was accompanied by an increase in CD36 in the skeletal muscles of diabetic animals. The decrease in β-arrestins and increase in CD36 was prevented by carvedilol treatment. Based on our results, metabolic effects by the carvedilol treatment seem to be due to downstream β-arrestin. However, carvedilol intrinsic vasodilatory effect as a third-generation β-antagonist in addition to its anti-oxidant and anti-inflammatory properties may also be involved in carvedilol's benefits in diabetes.

4.1 | Limitations

To avoid any interference of female sex characteristics and difficulty in the interpretation of the results, we conducted our experiments in male gender.

We had used C2C12 cells in the initial study where we reported that carvedilol decreased rates of fatty acid oxidation and stimulated glycolysis (Onay-Besikci, Suzmecelik, & Ozcelikay, 2012). In order to extrapolate the direct effects of carvedilol on substrate metabolism, we decided to stay on the same cell line. Although this cell line has been shown to mature into functional cardiac muscle cells (McMahon et al., 1994), they were not directed to contract or generate force for this study. We do acknowledge the fact that these "non-functioning" cells may have different characteristics in terms of substrate metabolism compared with a fully functional cardiomyocyte (Kolwicz, Purohit, & Tian, 2013).

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AUTHOR CONTRIBUTIONS

B.G., Z.K. and A.O.-B. performed the research. B.G. and A.O.-B. designed the research study. B.G., Z.K. and A.O.-B. analysed the data. B.G. and A.O.-B. wrote the paper.

CONFLICT OF INTEREST

None.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

All relevant data are within the manuscript and its Supporting Information files.

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